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An assessment of histone-modification antibody quality

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Wrote the manuscript: Thea Egelhofer, David Hawkins, Gary Karpen, Jason D. Lieb, Aki Minoda, and Susan Strome.

COMPETING INTERESTS STATEMENT

Fifteen non-commercial antibodies (indicated in Supplementary Table 1) were provided to us at no cost. We have no financial or material interest in the companies whose antibodies were tested.

Abstract

We report testing of the specificity and utility of over 200 antibodies raised against 57 different histone modifications, in *Drosophila melanogaster*, *Caenorhabditis elegans* and human cells. While most antibodies performed well, over 25% failed specificity tests by dot blot or western blot. Among specific antibodies, over 20% failed in chromatin immunoprecipitation experiments. We advise rigorous testing of histone-modification antibodies before use and provide a website for posting new test results.

INTRODUCTION

This December, we celebrate the 100th anniversary of Albrecht Kossel's 1910 Nobel Prize in Physiology or Medicine, which was awarded in part for his discovery of histone proteins¹. Soon after elucidation of the DNA-RNA-Protein 'Central Dogma', a 1964 paper presented strong experimental evidence that histones are acetylated and methylated after completion of the polypeptide chain, and that these histone modifications "affect the capacity of the histones to inhibit ribonucleic acid synthesis *in vivo*"². This work foreshadowed a very active period since the early 1990s, which has brought an explosion of insight regarding how DNA is packaged into chromatin, the multitude of enzymes that modify key histone residues in eukaryotic cells, and how those marks are associated with diverse functional states of chromatin³.

Key to these recent advances has been the availability of antibodies to dozens of specific post-translational modifications on histones, coupled with the advent of Chromatin Immunoprecipitation (ChIP), DNA microarrays (ChIP-chip), and highly parallel DNA sequencing (ChIP-seq). This combination of antibodies and technology has enabled investigators to determine the genomic distributions of histone modifications and to connect them with biological functions³. However, the reproducibility and biological relevance of histone-modification landscapes depends on the specificity and performance of the antibodies, most of which are now provided commercially. The validity of results could be affected by recognition of unmodified histones, non-target modifications, and non-histone proteins. In addition, antibodies may exhibit appropriate specificity, but be ineffective ChIP reagents.

Here we set out to assess the quality of histone-modification antibodies by western blot, dot blot, and ChIP-chip or ChIP-seq analysis.

RESULTS

As part of our activities in the NIH modENCODE⁴ and Roadmap Reference Epigenome⁵ initiatives, we performed three types of characterization on 246 antibodies directed against 3 unmodified histones and 57 distinct histone modifications (Supplementary Table 1). Western blot analysis was performed to test for cross-reactivity of the antibodies with unmodified histones or with non-histone proteins in nuclear or whole-cell extracts. Dot blots using a panel of modified peptides were used to test for cross-reactivity with other modifications. ChIP-chip or ChIP-seq⁶ was performed to test the ability of the antibodies to

reproducibly immunoprecipitate discrete DNA regions. The results are summarized below, with the details provided in Supplementary Table 1.

Western blot analysis

For fly and worm western blot analysis, a 3-fold dilution series of both total nuclear extract prepared from wild-type embryos and unmodified recombinant histone was electrophoresed on an SDS polyacrylamide gel, using an amount of recombinant histone that was comparable to the corresponding histone level in the nuclear extract (Fig. 1a). The following criteria were set for an antibody to pass: the histone band constituted at least 50% of the total nuclear signal, was at least 10-fold more intense than any other single nuclear band, and was at least 10-fold more intense relative to recombinant, unmodified histone. By these criteria, 80 of the 127 histone-modification antibodies tested (63%) passed, whereas 33 (26%) failed, and 14 (11%) produced no signal (Fig. 1b, c). For human western blots, whole-cell extract was used instead of nuclear extract. This resulted in a higher frequency of cross-reacting bands, many of which are likely to be irrelevant to assays performed on nuclear proteins. Therefore, we did not classify these as “pass” or “fail”, but described their behavior as shown in Fig. 1d.

Dot blot analysis

Dot blots were performed using a matrix of 43 peptides as shown in Fig. 2a, or on one of the arrays of peptides shown in Supplementary Fig. 1a, b. To pass, we required that at least 75% of the total signal be specific to the cognate peptide. According to this criterion, 109 of the 149 antibodies tested (73%) passed, of which 56% (61/109) displayed 100% specificity. Of the 149 tested antibodies, 20 (13%) produced signal on the dot blot but did not meet our specificity criterion, 16 (11%) yielded no signal, and 4 (3%) had low signal. (Fig. 2b). A particularly dangerous class of failure was defined by 4 antibodies (3%) that exhibited 100% specificity, but for the wrong peptide (Supplementary Table 1).

ChIP-chip and ChIP-seq analysis

We performed ChIP-chip or ChIP-seq experiments using 147 of the histone-modification antibodies, and judged them to have passed if they achieved a correlation of above 0.8 on any pair of ChIPs performed from independent preparations matched for stage, cell type, or biological tissue (Fig. 3a). This criterion only evaluates the effectiveness of the antibody to generate reproducible ChIP results, and does not measure whether the resulting distributions are biologically accurate. In the case of well-studied modifications, we were able to confirm that the signal conformed to previously established patterns, for example anti-H3K4me3 (histone H3 trimethyl lysine 4) precipitating chromatin near gene promoters³. In all, 115 of the 147 antibodies tested by ChIP (78%) passed, and 32 (22%) failed (Supplementary Table 1, Fig. 3b). Of the failures, 23 were marketed as ChIP-grade.

DISCUSSION

Our results show that most commercially available histone-modification antibodies perform well, but that at least 25% have substantial specificity or utility problems, suggesting that users should independently test purchased antibodies. Failure in one assay does not

necessarily predict failure in another, indicating that antibodies should be tested in multiple assays regardless of initial success or failure in a given assay. Manufacturers often provide peptide blot data, but assessment of cross-reactivity with non-histone proteins is usually restricted to one species, and the data presented are often based on lots that are no longer available for purchase. Substantial lot-to-lot variation (Supplementary Table 1) mandates that lots be tested separately using extracts from the species under study. Development of monoclonal antibodies to histone modifications may alleviate many of these concerns⁷. The high rate of specificity problems raises concerns about the validity of ChIP data generated and published without independent characterization.

To help address antibody quality issues in the community, we have developed an Antibody Validation Database website (<http://compbio.med.harvard.edu/antibodies/>) that allows researchers to post their assay results. This will provide up-to-date validation information, including tests of lot-to-lot variability. The website currently contains all histone-modification validation data described in this paper as well as data for other chromosomal proteins tested in the modENCODE project. The database can be searched by the modification or protein name, and it lists antibody details (source, catalog number, lot number, etc), links to the validation data including images, and other information such as the species and the laboratory in which testing was performed. Researchers publishing data generated with antibodies are encouraged to upload their validation information to this site.

METHODS

In addition to the websites below, all information about the antibodies is also listed at <http://compbio.med.harvard.edu/antibodies/>.

***C. elegans* nuclear extracts and western blotting**

C. elegans embryos, obtained by dissolving adult worms with bleach, were washed and dounce-homogenized 50 times using a tight pestle. Nuclei were collected by centrifugation and sonicated 2×30 min using a Branson sonicator to prepare extract. Samples in sample buffer were boiled, and a 3-fold dilution series of both nuclear extract and recombinant histone (purchased from Active Motif) were electrophoresed on a 12.5% SDS-polyacrylamide gel. The gel was stained with Coomassie blue to verify that approximately equal levels of recombinant histone and the corresponding histone were loaded. Samples were transferred to a nitrocellulose membrane. The membrane was blocked in non-fat milk, incubated with primary antibody, washed, incubated with secondary antibody, washed, and developed with ECL (Pierce). Western blot images are available at <http://www.modencode.org/docs/hmav.html>.

***D. melanogaster* nuclear extracts and western blotting**

D. melanogaster embryo nuclear extracts were prepared⁸. Three different dilutions of nuclear extract and recombinant histone (expressed in *E. coli*) were separated on an SDS-polyacrylamide gel. Western blot analysis was performed similarly to that described above.

Human western blotting

Whole cell extracts (WCE) were made from HCT116 cells. Samples were run on precast 4–20% gels and transferred to membrane. Western blot analysis was performed similarly to that described above. Detailed protocols and images of all blots can be downloaded from the San Diego Epigenome Center website: <http://epigenome.ucsd.edu>

Dot blots (Group 1)

Single-modification peptides were obtained from Abcam and Active Motif. The purity of peptides is 70–95%. Peptides were spotted onto nitrocellulose membrane in the pattern shown in Fig. 2a. The membrane was blocked in non-fat milk, incubated with primary antibody, washed, incubated with secondary antibody, washed, developed with ECL (Pierce), exposed in an imager and analyzed. Illuminated spots were encircled and quantitated. Percent-specificity is relative to total intensity of all illuminated modified-peptide spots normalized to background. Detailed protocols and images of all blots can be downloaded from the San Diego Epigenome Center website: <http://epigenome.ucsd.edu>

Dot blots (Group 2)

Slot/dot blot analysis was carried out according to standard protocols⁹ using nitrocellulose membrane and peptide (Diagenode) amounts ranging from 100 to 3 pmol. Diagenode claims that the purity of peptides is >70%. The intensities of the bands were analyzed by Image J, and percent-specificity was calculated relative to total intensity of all spots. For peptide array, see Supplementary Fig. 1a.

Dot blots (Group 3)

PVDF membranes (0.45 μ m pore size) were pre-washed in 100% methanol, rinsed 3 times in PBS and spotted with 100, 25 and 10 pmol of each peptide (Diagenode) in a 28-peptide matrix (Supplementary Fig. 1b). The membrane was allowed to dry, washed in 100% methanol, rinsed 3 times in PBS, blocked in 5% milk for 3 hours, and then incubated with antibody.

C. elegans ChIP-chip

ChIP-chip experiments were performed as described previously for early embryos¹⁰ and L3s¹¹.

D. melanogaster ChIP-chip

ChIP experiments were performed as previously described previously¹², with some changes. S2 *Drosophila* cultured cells were fixed in formaldehyde (Sigma) at a final concentration of 1.8% for 10 min. After several washes, the cells were homogenized using a dounce homogenizer, pelleted, resuspended in cold buffer, and SDS added to a final concentration of 1%. Cells were again pelleted, washed and finally resuspended at a final concentration of 1×10^8 nuclei/ml, with 0.1% SDS. Cells were sonicated using a Bioruptor sonicator. All lysates were combined, after which Triton-X 100 and Deoxycholate were added. After centrifugation, the final supernatant contained soluble chromatin. Input chromatin was treated with RNase, followed by Proteinase K, and crosslinking was

reversed. The average size of the DNA fragments was 400–1000 bp. For ChIP, chromatin was pre-cleared by incubating with Protein A Sepharose beads. After the beads were removed, chromatin was incubated with the antibody for immunoprecipitation, followed by addition of Protein A Sepharose beads. After washing, sample attached to beads was treated with RNase A, followed by Proteinase K, and crosslinking was reversed. Half of each ChIP sample and 50 ng of Input DNA were amplified using a WGA Kit (Sigma # WGA2). Samples were purified using a QIAquick PCR purification column (QIAGEN). The amplified DNA was fragmented using RNase-free DNaseI, after which the peak of bulk DNA was at 50–100 bp. The fragmented DNA library was labeled with biotin by a terminal deoxynucleotidyl transferase reaction, and hybridization cocktail was added. Genomic DNA Tiling Arrays v2.0 (Affymetrix) were pre-hybridized and then hybridized to ChIP sample or Input DNA for 18 hours, followed by washing and staining in a fluidics station EukGE-WS2v4 (Affymetrix). Enrichment P-values are calculated using a sliding window (default size 1 kb) moved in steps across the genome (default step size 30 bp). A P-value enrichment score is calculated at each step using a one-sided T-test on the M-values of probes that fall within the window. To capture both significant enrichment and significant depletion, P-values for enrichment test (ePv) and depletion test (dPv) are calculated, and the score is given as $-\log_{10}(\min(ePv, dPv))$. The score is multiplied by -1 if dPv was smaller than ePv.

Human ChIP-chip and ChIP-seq

Procedures for ChIP-chip and ChIP-seq have been described previously¹³. For ChIP-chip, enrichment was defined as a >2 -fold change and $p < 0.001$. For ChIP-seq, enrichment was defined as previously described, and biological replicates were correlated¹³.

Equipment and Settings

Coomassie staining and western blots (Fig. 1a) were scanned and then scaled and labeled using Photoshop. Dot blot images (Fig. 2a) were captured, processed and analyzed using the Alpha Innotec FC2 imager. The FC2 software was used to grid images and enumerate spots for dot blots.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

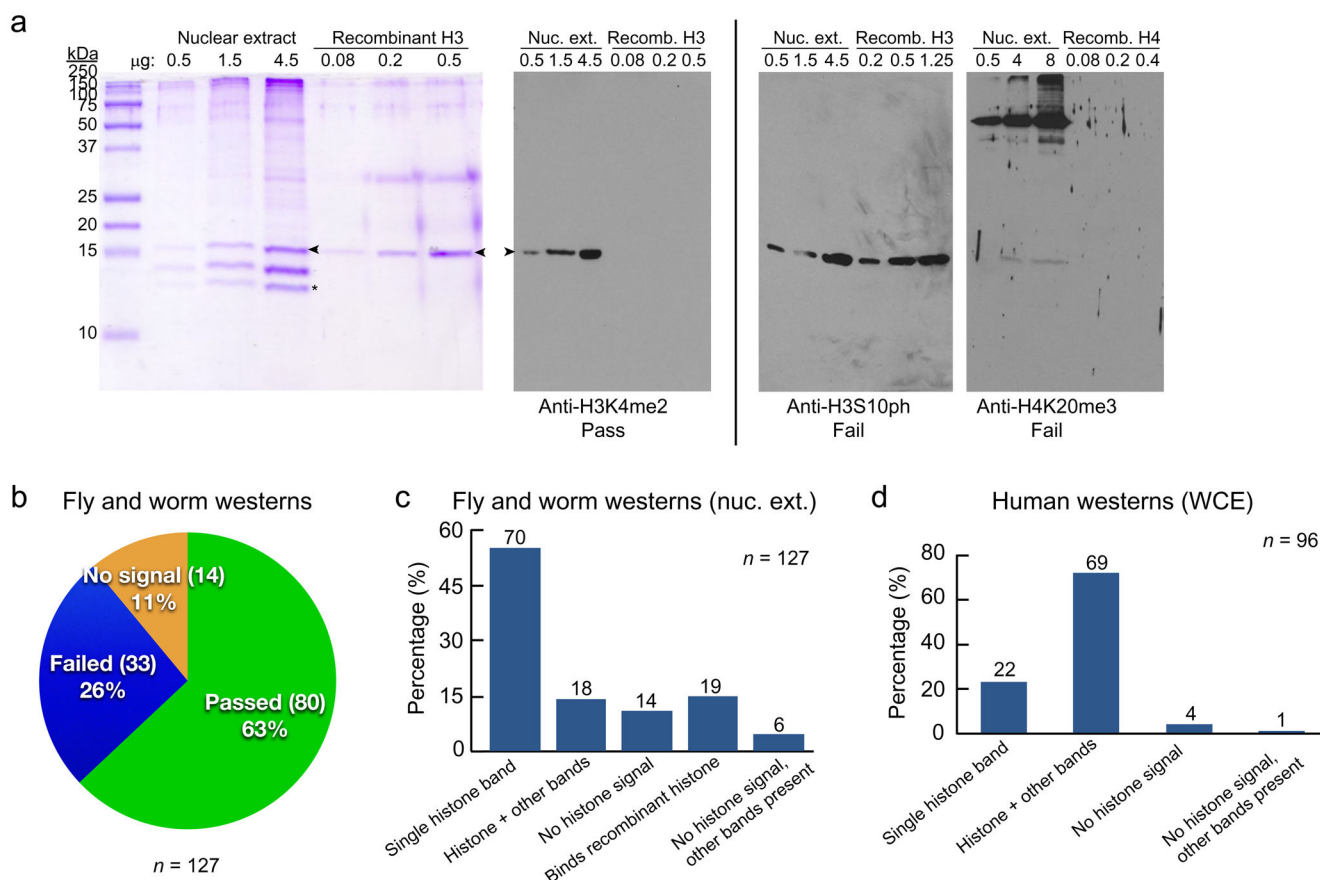
Acknowledgments

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**Figure 1.**

Representative western assays and results. (a) Western blot of anti-H3K4me2 (Millipore, 07-030, lot DAM1543701), anti-H3S10ph (Wako, 303-35199), and anti-H4K20me3 (Diagenode, CS-057, lot A9-002). Left panel shows Coomassie blue stained gel of worm nuclear extract and recombinant H3 (Active Motif, 31207), showing the amount of protein loaded in each lane and approximately equal levels of histone H3 in the nuclear extract and recombinant H3 sets of lanes. Histone H3 is marked with an arrowhead and histone H4 with an asterisk. Anti-H3K4me2 passed, because it recognized only H3 in the nuclear extract and not unmodified H3. Anti-H3S10ph failed, because it recognized unmodified H3 with equal intensity to H3 in the nuclear extract. Anti-H4K20me3 failed, because it recognized non-histone proteins and perhaps H3 instead of H4 in nuclear extract. All western blot images are available at <http://compbio.med.harvard.edu/antibodies/>. Images are also available at <http://www.modencode.org/docs/hmav.html> (worm and fly) <http://epigenome.ucsd.edu/antibodies.html> (human). (b) Summary of fly and worm western blot results. Antibodies against core histones were not included in the summation, since they are expected to detect recombinant histones. Test results differed among groups for 3 antibodies (pass versus no signal, or fail versus no signal). Those antibodies were included in the pass or fail categories, respectively. (c) Performance of antibodies tested in fly and worm nuclear extracts (nuc. ext.). Antibody results were binned into 5 mutually exclusive groups; the percentage is plotted, with the number of antibodies shown above each bar. Same exceptions

were applied as in panel b. (c) Performance of antibodies tested in human whole-cell extracts (WCE). Many antibodies classified as “Histone + other bands” passed ChIP tests.

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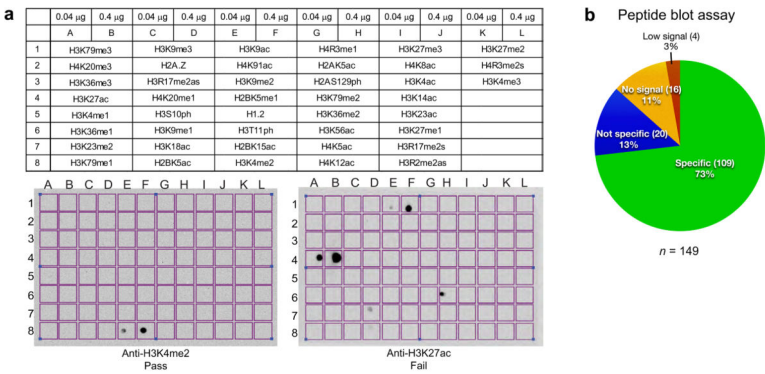
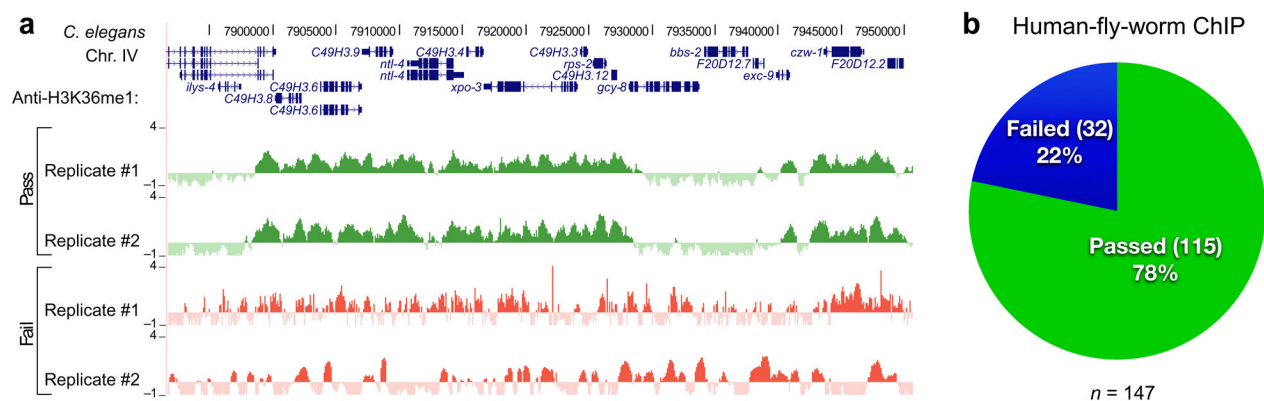


Figure 2. Representative dot blot assays and results. (a) Dot blot characterization of anti-H3K4me2 (Abcam, ab32356, lot 577702) and anti-H3K27ac (Abcam, ab4729, lot 726657). Top panel shows the positions of histone tail peptides spotted on membranes. Anti-H3K4me2 passed. Anti-H3K27ac failed due to detection of multiple peptides. Human, fly, and worm dot blot images are available at the web sites noted in the legend to Figure 1. (b) Summation of peptide blot results. 149 antibodies were classified as described in the text. Low signal indicates that only the highest peptide concentration was detected by the antibody. See Supplementary Fig. 1 for a description of the peptide array used for each antibody, and Supplementary Table 1 for enumeration of cross-reacting peptides.

**Figure 3.**

ChIP-chip and ChIP-seq. (a) Representative ChIP-chip characterization. Shown is anti-H3K36me1 from two different sources (Abcam, ab9048 lot #18882 and H. Kimura, 1H1). A ~60 kb region of *C. elegans* chromosome IV is shown, with annotated genes (X-axis) and ChIP-chip z-scores (standardized \log_2 ratios of ChIP/Input signals) (Y-axis) plotted for biological replicates using both antibodies. The replicates were highly correlated using the Abcam antibody (passed), but not using the 1H1 antibody (failed). (b) Summary of results. Antibodies against core histones were not included in the summation.